*Biochem. J.* (1971) **125**, 917–919
*Printed in Great Britain*



# The Identification of Monoconjugates of Bilirubin in Bile as Amide Derivatives

By F. H. JANSEN* and BARBARA H. BILLING
*Department of Medicine, The Royal Free Hospital, London WC1X 8LF, U.K.*

(*Received* 27 *September* 1971)

Recent reports have shown that bilirubin can be esterified *in vivo* to form a variety of acyl glycosides (Kuenzle, 1970; Heirwegh, Van Hees, Leroy, Van Roy & Jansen, 1970). The question as to whether these conjugates are present in bile as monoconjugates as well as diconjugates remains in doubt. Indirect evidence for the existence of bilirubin monoglucuronide, based on the estimation of the molar ratio of bilirubin to glucuronic acid and on the production of equal amounts of azobilirubin and of azobilirubin glucuronide on diazotization of an isolated bilirubin conjugate, has been presented by several groups of workers (Billing, Cole & Lathe, 1957; Giovanetti, Maggiore & Vivaldi, 1961; Jacobsen, 1969; Ostrow & Murphy, 1970). A direct approach to the problem of mono-conjugation has, however, been hampered by the complexity and instability of bilirubin conjugates as well as the failure of investigators to isolate them in a chemically pure form. In order to overcome these difficulties a search was made for a substitution reaction that was specific for esters, so that derivatives could be prepared that were suitable for analysis by physicochemical techniques. Information as to which carboxyethyl side chain of bilirubin was involved in conjugation would then be indicated by the position of the substituent in the derivative.

Ammonolysis of conjugated bilirubin proved to be an acceptable procedure since, under suitable conditions, bimolecular nucleophilic substitution (Sykes, 1965) occurs at the carbonyl carbon atom of the esterified carboxyethyl side chain of bilirubin, and bilirubin amides are formed. The reaction is very quick, owing to the alkali-lability of the glycosidic bond, and is independent of whether the conjugate is a glucuronide or an acidic disaccharide.

Preparations of bilirubin conjugates that contained minimal amounts of unconjugated bilirubin and other bile constituents were prepared from human bile or from rat bile (after a loading dose of bilirubin) by the method of Lucassen (1961) as described by Jacobsen (1969). An estimate of the relative amounts of mono- and di-conjugates present was made from an analysis of their ethyl anthranilate azopigment composition (Ostrow & Murphy, 1970).

Approx. 5mg of mixed bilirubin conjugates, dissolved in 0.1ml of water, was added to 4ml of aq. ammonia (sp.gr. 0.88), mixed for 30s and then immediately transferred to a separating funnel containing 100ml of glycine–HCl buffer, pH 2.8 (1.2mol of HCl/l, 4.1mol of glycine/l), and 100ml of chloroform, and vigorously shaken so as to stop the reaction and extract the pigments. After separation the chloroform layer, containing most of the pigments, was concentrated *in vacuo*, and a portion was applied to silica-gel plates (DC.-Alufolien 5553/0025; E. Merck A.-G., Darmstadt, Germany) and developed over 15cm in chloroform–methanol (49:1, v/v). Four distinct chromatographic bands were obtained, together with a small quantity of the original bilirubin conjugates, which remained at the origin. The four pigment bands were immediately eluted from the silica gel with chloroform and evaporated to dryness; in this form they appeared to be stable. The pigments were examined by mass spectrometry (Fig. 1) and, in order of decreasing $R_F$ values on the chromatogram, were identified as bilirubin, two bilirubin monoamides (I and II) and bilirubin diamide. Analysis of the molecular ions by accurate mass measurement gave values of 583.2787 (Calc. for $C_{33}H_{37}N_5O_5$ : 583.2794) for each of the monoamides and 582.2944 (Calc. for $C_{33}H_{38}N_6O_4$ : 582.2954) for the diamide compared with 584.2634 (Calc. for $C_{33}H_{36}N_4O_6$) for bilirubin.

The two bilirubin monoamides were converted into ethyl anthranilate azopigments as previously described for bilirubin (Jansen & Stoll, 1971). T.l.c. on silica-gel plates in chloroform yielded approximately equal amounts of azobilirubin and azobilirubin amide, which were identified by mass spectrometry. The azobilirubin moiety obtained from each monoamide was converted into its methyl ester by treatment with diazomethane and its vinyl–isovinyl isomeric composition was established by chromatography in chloroform–benzene (1:1, v/v) (Jansen & Stoll, 1971). The isovinyl isomer of ethyl anthranilate azobilirubin methyl ester was the only product obtained from bilirubin monoamide I. It can be concluded that its complementary azobilirubin amide moiety has a vinyl

* Present address: Department of Medicine, Academisch Ziekenhuis, Leuven 3000, Belgium.

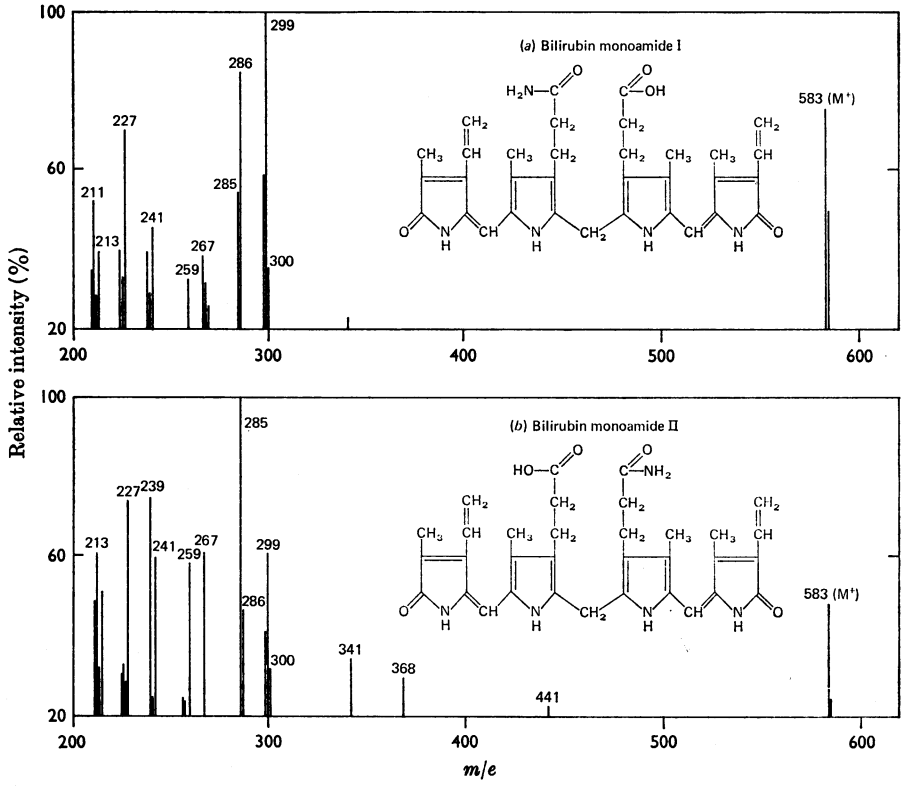


Fig. 1. Normalized histograms of the mass spectra and chemical structures of the isomeric bilirubin monoamides. The mass spectra were recorded by Mr D. Carter of the University of London Mass Spectrometry Service, at the School of Pharmacy, on an MS902 double-focusing mass spectrometer (A.E.I., Manchester, U.K.). The samples were inserted on a direct probe. The temperature of the ion source was 250°C. The spectra were run at an ionizing voltage of 70 eV. For the bilirubin monoamides intense molecular ions were recorded at *m/e* 583. The main fragmentation results from fission at the central methylene bridge of the bilirubin molecule, yielding intense ions at *m/e* 300 and 299 and at *m/e* 286 and 285. The ions at *m/e* 299 and 285 are consistent with that half of the bilirubin molecule possessing the amide group. Other ions are identical with those found for bilirubin and arise mainly from the fragmentation of the carboxyethyl or carbamide-ethyl side chain. The ion at *m/e* 267 presumably results from the loss of water from the amide group of the ion at *m/e* 285. This phenomenon was also observed in the mass spectrum of ethyl anthranilate azobilirubin amide, where the molecular ion at *m/e* 461 loses water, yielding an ion at *m/e* 443. This rearrangement is unusual for amides, and it is probably the result of a thermic effect as it did not occur when the sample was recorded at 200°C. In the mass spectrum of bilirubin diamide a weak molecular ion was observed at *m/e* 582; further fragmentation was similar to that observed for all rubins.

isomeric structure, since equal amounts of the vinyl and isovinyl azobilirubin isomers were obtained from the original conjugated bilirubin preparation after diazotization of the bilirubin conjugates and methylation of the azobilirubin chromophores. In contrast, only the vinyl azobilirubin methyl ester was obtained from the more polar monoamide (II), so that the amide half of that bilirubin monoamide must have an isovinyl configuration. The structures indicated in Fig. 1 have therefore been assigned to bilirubin monoamides I and II.

T.l.c. of the azopigments obtained from bilirubin diamide showed that only ethyl anthranilate azobilirubin amide was formed. No attempt was made to separate and identify the vinyl and isovinyl isomers.

Although the formation of bilirubin amides from bilirubin does not occur under the above conditions, some base-catalysed hydrolysis of diconjugates and/or diamides, yielding monoamides or unconjugated bilirubin, is inevitable. It has been found, however, that bile pigment preparations containing

approx. 80% diconjugates (as judged by their ethyl anthranilate azopigment pattern) yielded predominantly bilirubin diamides (44–66% of the total of pigments separated by t.l.c.). From rat biles, containing 85% monoconjugates, approx. 65% of the pigments obtained after ammonolysis were bilirubin monoamides. The two isomeric forms were found to be present in approximately equal quantities. The amount of unconjugated bilirubin formed ranged from 6 to 21% of the total pigments. These results are in contrast with the fast hydrolysis of bilirubin conjugates that occurs with dilute alkali.

Since the reaction employed for the preparation of the amides is specific for esters, these results show that in human and rat bile both mono- and diconjugates of bilirubin occur and that the monoconjugates are excreted in two isomeric forms. If the major excretory pathway for bilirubin involves conjugation as a simple glucuronide (Billing & Jansen, 1971), then this study could be interpreted as indicating that bilirubin monoglucuronide exists as a chemical entity in bile.

F. H. J. thanks the Wellcome Trust for financial support. Requests for reprints should be addressed to B. H. B.

Billing, B. H., Cole, P. G. & Lathe, G. H. (1957). *Biochem. J.* **65**, 774.

Billing, B. H. & Jansen, F. H. (1971). *Gastroenterology*, **61**, 258.

Giovanetti, S., Maggiore, Q. & Vivaldi, G. (1961). *Ital. J. Biochem.* **10**, 88.

Heirwegh, K. P. M., Van Hees, G. P., Le Roy, P., Van Roy, F. P. & Jansen, F. H. (1970). *Biochem. J.* **120**, 877.

Jacobsen, J. (1969). *Acta chem. scand.* **23**, 3023.

Jansen, F. H. & Stoll, M. S. (1971). *Biochem. J.* **125**, 585.

Kuenzle, C. C. (1970). *Biochem. J.* **119**, 411.

Lucassen, J. (1961). Doctoral Thesis: University of Utrecht.

Ostrow, J. D. & Murphy, N. (1970). *Biochem. J.* **120**, 311.

Sykes, P. (1965). *A Guidebook to Mechanism in Organic Chemistry*, pp. 58–67, 184. London: Longmans, Green and Co. Ltd.